Stereoselective Pharmacokinetics of Ifosfamide in Male and Female Rats

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ABSTRACT The stereoselective pharmacokinetics of ifosfamide (IF) were investigated in male and female Sprague-Dawley rats. Following intravenous administration of IF deuterium-labeled pseudoracemates into rats at 40 mg/kg, IF enantiomers and their metabolites, 4-hydroxyIF (HOIF), N^2-dechloroethylIF (N2D), N^3-dechloroethylIF (N3D), and isophosphoramide mustard (IPM) were quantitated in plasma and urine using gas chromatographic-mass spectrometry techniques with appropriately deuterium-labeled analogs as the internal standards. In addition, the intrinsic clearances of IF isomers in rat liver microsomes were estimated by the in vitro metabolism study. Following drug administration in male rats, (*R*)-IF exhibited a lower area under the curve value and a shorter half-life of 34.2 minutes than (*S*)-IF, which gave a half-life of 41.8 minutes. In female rats, the half-lives of (*R*)- and (*S*)-IF were found to be 62.1 and 75.1 minutes, respectively, significantly longer than those in male rats. No change in volume of distribution or renal clearance for IF enantiomers in all rats was observed, and the protein binding value was low, with no enantioselectivity. Both in vitro and in vivo studies showed that metabolism of (*R*)-IF proceeded in favor of the 4-hydroxylation pathway, whereas (*S*)-IF preferentially underwent N^2- and N^3-dechloroethylation. The observed stereoselectivity and gender difference in pharmacokinetics of IF in the rat are mainly attributed to its stereoselective metabolism.

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INTRODUCTION

Ifosfamide (IF) is a widely used anticancer prodrug (1-2). Its detailed metabolic pathways have been reported recently (3) (Scheme 1). IF undergoes 3 primary metabolic transformations in the liver, generating 4-hydroxyifosfamide (HOIF), N^2-dechloroethylifosfamide (N2D), and N^3-dechloroethylifosfamide (N3D). The generated HOIF is further decomposed into isophosphoramide mustard (IPM), which is believed to be the ultimate alkylating metabolite. IF is a chiral drug, and 2 enantiomers differ in efficacy and toxicity (4-6). The disposition of IF appears to be stereoselective as well (6-11). Stereoselectivity in pharmacokinetics of a chiral drug has generally been attributed to the process of absorption, distribution, and/or hepatic and renal elimination (12-23). Although it has been found that IF enantiomers possess different pharmacokinetic profiles, reasons for the difference in drug disposition between 2 enantiomers have not been fully evaluated. Stereoselectivity in metabolism of IF has been investigated, but previous studies focused mainly in the N-chloroethylation pathways (9,14-19). It was shown that (*S*)-IF metabolism favors of dechloroethylation in humans and rats.

N-2 and N-3-dealkylations represent 2 of the major metabolic pathways of IF and generate metabolites that are devoid of antitumor activity, although the coproduct chloroacetaldehyde has been implicated in the neurotoxicity of IF (20). The stereoselectivity of the activation pathway, hydroxylation at C-4 of IF, has not been investigated. Our in vivo pharmacokinetic studies using pseudoracemate indicated that, in the uninduced rat, there was preference in the production of (R)-4-hydroxyIF, as evidenced by the area under the curve (AUC) of the

metabolite, and phenobarbital reversed the stereochemical preference (21). IF metabolism has been shown to be catalyzed by a number of cytochrome P450 isozymes (24). These enzymes distribute differently among species and between sexes. Because the rat model has been used in pharmacokinetic and metabolism study of IF in many settings, a thorough understanding of the pharmacokinetics and metabolism in this species is essential. Thus, the present study expands the stereochemical investigation of IF in the uninduced rat in all major metabolic pathways by including the in vitro metabolic studies and also by examining the sex difference.

METHODS

Chemicals

(*R*)- and (*S*)-IF, (*R*)- and (*S*)-6,6,2',2'-tetradeuterioIF, [(*R*)- and (*S*)-IF-d$_4$] (Figure 1) were synthesized in this laboratory (22). The isotope purity was greater than 99.0%, as determined by gas chromatographic-mass spectrometry (GC/MS) analysis. The metabolites, HOIF, N2D, N3D, IPM, and their respectively labeled internal standards, 4,4,5,5,6,6-hexadeuterioIF (IF-d$_6$), 4-hydroperoxy 6,6,2',2',2",2"-hexadeuterioIF (HOOIF-d$_6$), N^2-dechloroethyl-1',1',2',2'-tetradeuterioIF (N2D-d$_4$), N^3-dechloroethyl-4,4,6,6,1',1',2',2'-octadeuterioIF (N2D-d$_8$), 2',2'-dideuterioiphosphoramide mustard (IPM-d$_2$), and 1',1',2',2',1",1",2",2"-octadeuterioiphosphoramide mustard (IPM-d$_8$), were all synthesized in this laboratory (23). The chemical purity of the synthetic compounds was larger than 98%. The internal standard, 4-hydroxy-6,6,2',2',2",2"-hexadeuterioIF (HOIF-d$_6$), was prepared by the reduction of HOOIF-d$_6$ with sodium thiosulfate immediately before use. IF pseudoracemate was prepared by mixing equal amounts of (*R*)-IF and (*S*)-IF-d$_4$ or (*R*)-IF-d$_4$ and (*S*)-IF. High-performance liquid chromatography grade dichloromethane and methanol were purchased from Fisher Scientific (Pittsburgh, PA). N-methyl-N-trimethylsilytrifluoroacetamide (MSTFA), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), and N-trimethyl-silylimidazole (TMSI) were obtained from Pierce (Rockford, IL). C-18 reversed-phase

resin was obtained from Analytichem International (Harbor City, CA). NADP$^+$, D-glucose-6-phosphate, and yeast glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO).
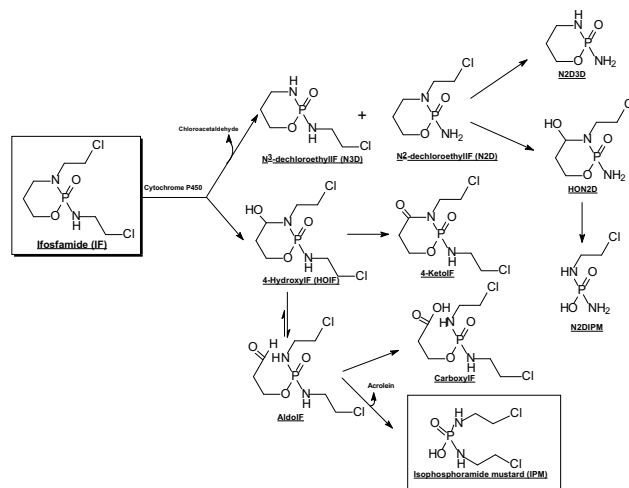
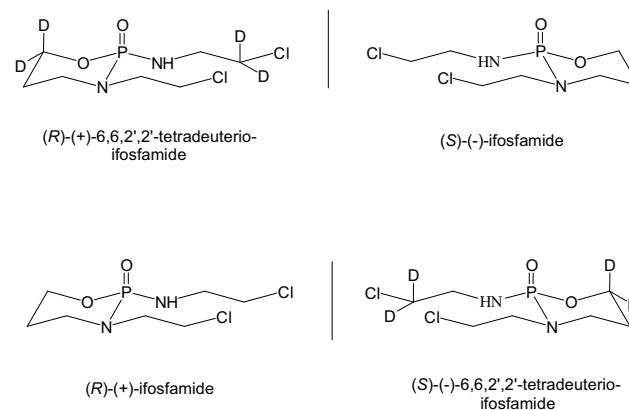**Scheme 1: The Major Metabolic Pathways of Ifosfamide (IF).**

Figure 1. Chemical structures of pseudoracemates of IF[(R)-IF-d4/(S)-IF and (R)-IF/(S)-IF- d4, 1:1] in this study.

Pharmacokinetic Study

Six male and 5 female Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 280 to 330 g were used in this study. The left jugular and femoral veins of each rat were cannulated under ether anesthesia (25). Following surgical manipulation, the animals were

allowed to recover for at least 1 hour before drug dosing. IF pseudoracemate at a total dose of 40 mg/kg in 0.3 mL saline was administered to each rat through the femoral vein cannula, followed by flushing each cannula 3 times with 0.1 mL of saline. Three male and 3 female rats were given the pseudoracemate pair of (R)-IF-d_4/(S)-IF, and another 3 male and 2 female rats were given the pseudoracemate pair of the opposite configuration. Rat chow (Tekland, Indianapolis, IN) and water were given ad libitum. Blood samples (0.2-0.4 mL each) were collected from the jugular vein cannula at 5, 15, 30, 45, 60, 75, 90, 120, 180, 240, 300, 360, and 420 minutes postdose and placed into heparinized culture tubes immersed into an ice-bath. A 36-hour cumulative urine sample was also collected. Blood samples were centrifuged at 1000 g for 5 minutes at 4°C, and plasma was then separated. Plasma and urine samples were frozen at -70°C until analysis.

Evaluation of Possible Isotopic Effect

Two male Sprague-Dawley rats (Harlan, Indianapolis, IN) were used to evaluate the possible isotopic effect of the labeled pseudoracemates. (R)-IF/(R)-IF-d_4 (1:1) or (S)-IF/(S)-IF-d_4 (1:1), at a total dose of 40 mg/kg in 0.3 mL saline, was administered into the rat through the femoral vein cannula, followed by flushing 3 times with 0.1 mL saline each. Rat chow (Tekland, Indianapolis, IN) and water were given ad libitum. Blood samples (0.3 mL each) were collected from jugular vein cannula using the sampling schedule described above. Blood and cumulative urine samples were processed as before.

Partition of IF Enantiomers Between Red Blood Cells and Plasma

Fresh rat blood was obtained from male Sprague-Dawley rats. A preliminary experiment to determine the time-course of drug uptake into red blood cells (RBC) was first carried out at 25 µM IF following incubation times of 5, 15, and 30 minutes. An appropriate amount of IF pseudoracemate [(R)-IF/(S)-IF-d_4] was added to freshly obtained blood to result in final respective concentrations of 25 and 100 µM. The mixture was incubated at 37°C for 15 minutes. Immediately afterward, each blood sample was

divided into 2 portions, one for determination of IF blood concentration (C_b) (26) and the other for plasma concentration (C_p). For the latter, plasma was obtained following centrifugation at 1000 g for 5 minutes. Five replicates for each concentration were performed. The ex vivo partition experiment of IF enantiomers was carried out as follows. IF pseudoracemate [(R)-IF/(S)-IF-d_4] at 40 mg/kg was injected into a rat. Blood samples at 0.3 mL each were collected at 2, 5, 15, 30, 45, 60, 75, 90, 120, 180, 240, and 300 minutes postdose and separated into 2 portions, one for C_b and the other for C_p analyses as before. Concentrations of IF enantiomers in RBC (C_{rbc}) were estimated from C_b, C_p, and blood hematocrit (H_t: 0.45) according to the following equation (27):

$$C_{rbc}=\{C_b-[C_p(1-H_t)]\}/H_t \qquad \text{[Equation 1]}$$

Protein Binding Study

The binding of IF enantiomers to rat plasma proteins was determined using the equilibrium dialysis method. Spectra/por 6 dialysis bags (MWCO 1000 Daltons, 1.15 8 cm each) (Fisher Scientific, Pittsburgh, PA) containing 1 mL rat plasma each were tightly ligated at both ends and placed in 10 mL of 0.067 M, pH 7.4 sodium phosphate buffer saline containing an appropriate amount of IF pseudoracemate [(R)-IF/(S)-IF-d_4]. The entire assembly was allowed to equilibrate for 4 days in a 4°C refrigerator. Then IF concentrations in buffer and plasma were determined. The free fractions of IF enantiomers in plasma were estimated by dividing the concentration of each enantiomer in buffer (free) to that in plasma (total). A duplicate experiment was performed at each concentration. Only protein binding in male rat plasma protein was performed.

In Vitro IF Metabolism in Liver Microsomes

This study aimed to compare the metabolic activities toward IF in male and female liver microsomes. Microsomes were prepared using the differential ultracentrifugation method (28). Briefly, male or female Sprague-Dawley rats (280-330 g) were killed after an overnight fast. Liver was removed and washed with ice-cold 1.15% (w/v) KCl in pH 7.4,

0.05 mol sodium phosphate buffer, then minced into small pieces in 4 volumes of the same buffer. The mixture was homogenized with 4 passes in a Potter-Elvehjem homogenizer, then centrifuged at 10,000 g for 20 minutes. The pellet was resuspended in the same phosphate buffer and centrifuged at 10,000 g for 20 minutes. The supernatants from both centrifugations were combined and further centrifuged at 100,000 g for 60 minutes at 4°C. The final pellet was suspended in 0.1 mol sodium phosphate buffer pH 7.4 containing 20% (w/v) glycerol and 0.1 mmol EDTA and used to study the metabolism of IF. Protein concentration was determined by the Lowry method using bovine serum albumin as a standard (28).

The in vitro drug metabolic studies in the liver microsomes as obtained above were carried out in uncapped 2-mL polypropylene microtubes. A mixture typically consisted of 0.5 to 1 mg microsomal protein, various concentrations of IF from 0.25 to 4 mmol, 15 mmol $MgCl_2$, 1.2 mmol $NADP^+$, and 10 mmol D-glucose-6-phosphate; 2 units/mL yeast glucose-6-phosphate dehydrogenase in a 0.2-mL, 0.1-mol, pH 7.4 sodium phosphate buffer was also incubated at 37°C for 15 minutes. The conditions that yielded linearity between rates of metabolite formation versus time (protein amount held constant) and microsomal concentrations (time held constant), using substrate IF at 1 mmol from several trials, were selected. The reaction was initiated by an addition of an NADPH- generating system after 3 minutes preincubation and terminated by an addition of 1 mL ice-cold methylene chloride. The samples were then stored at -80°C until analysis.

GC/MS Analyses

The analysis of IF enantiomers and their metabolites was accomplished using the GC/MS method developed in this laboratory (23,29). Briefly, plasma and urine samples were thawed at 0 to 5°C, and each was divided into 2 portions. The portion for the analysis of IF, HOIF/aldoIF, N2D, and N3D was immediately placed in a culture tube containing 200 μL of 1.5-mol pH8 KCN solution; 1 μg of IF-d_6, N2D-d_4, and N3D-d_8; and 2 μg of HOIF-d_6. The samples were allowed to stand at room temperature

for 30 minutes, followed by an addition of 5 mL of methylene chloride. The mixture was shaken for 15 minutes. After centrifugation, the organic phase was separated. Following evaporation of the organic solvent to dryness under a stream of nitrogen, the residue was derivatized with 40 μL of MSTFA at 120°C for 1 hour. A 1-μL aliquot of the derivatized sample was injected into the GC/MS.

The other portion of the sample was used for the analysis of IPM. After addition of 1.5 μg of IPM-d_8, the plasma was mixed and placed onto a disposable poly-prep minicolumn (Bio-Rad, Richmond, CA) containing 400 mg C18 reversed-phase resin. The resin was washed with 1 mL of ice-cold saline followed by centrifugation at 1500 g for 10 minutes to remove as much water as possible. The mini-column was then eluted with 1 mL of methanol, and the methanol extract was evaporated to dryness under nitrogen. The residue was derivatized with 40 μL of a mixture of BSTFA and TMSI (5:1, vol/vol) at 120°C for 40 minutes, and 1 μL of aliquot was injected into the GC/MS.

GC/MS analysis of derivatized IF, metabolites, and their internal standards was carried out on a Finnigan ITS40 ion-trap mass spectrometer (Finnigan MAT, San Jose, CA) directly coupled to a 300/3400 Varian gas chromatograph (Walnut Creek, CA) via a capillary splitless injector. The mixture was separated on a DB-5 fused-silica capillary column (30 m 0.25 mm I.D.) bonded with a 0.25-μm film thickness of 5% methylsilicone (J & W Scientific, Folsom, CA). Helium was used as the carrier gas with a head pressure set at 15 psi. Chemical ionization mode was used with ammonia as the reagent gas. The temperatures of the injection port, transfer line, and source were set at 220, 260, and 230°C, respectively. The initial oven temperature was set at 150°C for 2 minutes and increased to 190°C at a rate of 5°C/minute, and then to 250°C at a rate of 15°C/minute. The final temperature was held for 3 minutes. The ions with m/z ratios of 225, 229, and 233 for unlabeled IF, labeled IF-d_4, and their internal standards (IF-d_6); 235, 237, and 239 for N2D, N2D-d_2 and its internal standard (N2D-d_4); 235, 239, 243 for N3D, N3D-d_4 and its internal standards (N3D-d_8); 412, 416, and 420 for HOIF, HOIF-d_4, and its

internal standard (HOIF-d$_6$); 329, 333, and 337 for IPM, IPM-d$_2$, and its internal standards (IPM-d$_8$) were detected, respectively.

Data Analysis

Regression analysis and pharmacokinetic model fitting were accomplished using the PCNonlin program (Statistical Consultants, Lexington, KY) on an IBM PC. An appropriate compartmental model was selected based on the Akaike's information criterion (AIC) and the smallest value of the weighted sum of square. Pharmacokinetic parameters such as total drug clearance (CL$_T$), mean residence time (MRT), and steady-state volume of distribution (V$_{ss}$) were calculated as follows:

$$Cl_T = D/AUC \qquad \text{[Equation 2]}$$

$$MRT = AUMC/AUC \qquad \text{[Equation 3]}$$

$$V_{ss} = MRT \times Cl_T \qquad \text{[Equation 4]}$$

where AUC and AUMC are the area under the concentration-time curve and area under the first moment curve, respectively. The trapezoidal rule was used to estimate AUC and AUMC values and extrapolated to time infinity. The apparent K_m and V_{max} values of the in vitro hepatic metabolism were estimated using a nonlinear Michaelis-Menten regression. Statistical analysis was performed by analysis of variance and paired t test. In most cases, the significant level was set at $P = .01$.

RESULTS

Evaluation of Isotopic Effect

Concentration-time profiles of (R)-IF or (S)-IF and their metabolites following simultaneous intravenous administration of unlabeled and labeled enantiomers into the rat at 20 mg/kg each are shown in Figure 2. No significant difference in plasma drug concentrations was found between labeled and unlabeled (R)- or (S)-IF enantiomer. Thus, no appreciable isotopic effect at the labeling positions at 6- and 2'- positions was found.

Partition of IF Enantiomers Between RBC and Plasma in Rat Blood

Initially, a preliminary study of IF in rat blood at 25 μM was carried out to ascertain the optimal time of incubation, and it was found that the plasma and whole blood concentrations of enantiomers were not significantly different following 5, 15, or 30 minutes incubation. Therefore, an incubation time of 15 minutes was used for all subsequent studies. When IF pseudoracemate at 25 μM and 100 μM was separately incubated with rat blood, the plasma-to-blood concentration ratios (C$_p$/C$_b$) for (R)-IF were found to be 1.10 ± 0.04 and 1.07 ± 0.07; for (S)-IF they were 1.08 ± 0.05 and 1.05 ± 0.05 (all n = 5), respectively.
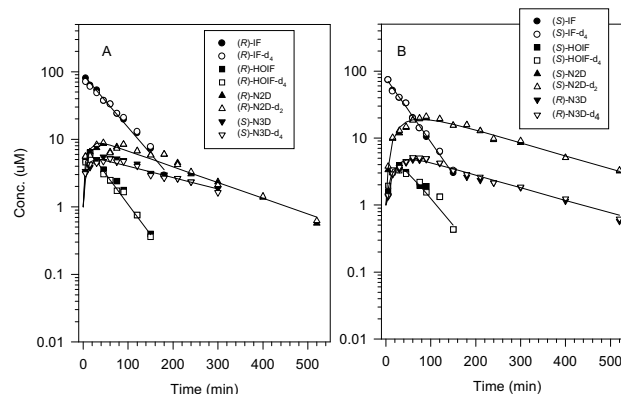

Figure 2. Concentration-time profiles of IF enantiomers and their metabolites after intravenous administration of labeled and unlabeled single enantiomer®-IF (a) or (S)-IF (B) into rats at a total dose of 40 mg/kg.

The ex vivo partition study for the individual enantiomer was also carried out, and the mean C$_p$/C$_b$ values for (R)- and (S)-IF across 2 to 300 minutes were 1.12 ± 0.06 and 1.07 ± 0.08 (n = 12), respectively. Thus, no significant difference in partition of 2 enantiomers between RBC and plasma was observed. Thus, there was no stereoselective distribution of IF enantiomers into RBC in rat blood.

Protein Binding of IF

Protein binding rates for 2 enantiomers in male rat plasma were found to be low and similar. The mean free fractions for (R)- and (S)-IF were found to be 15.7 ± 5.4% and 16.5 ± 5.5% (n = 6), respectively, across the concentration range from 6 to 100 μM. Additionally, in this concentration range, free plasma

concentrations of (R)- and (S)-IF were proportional to their total concentrations, indicating no saturation in protein binding. Thus, no stereoselective protein binding was found for IF enantiomers. Based on this result, the protein binding is not likely to be important in modulating the disposition of IF. Therefore, the protein binding of IF in female rat plasma protein was not studied.

Pharmacokinetics of IF Enantiomers in Male and Female Rats

Following intravenous administration of IF pseudoracemate to male and female rats at a total dose of 40 mg/kg, plasma concentration-time profiles of IF enantiomers declined monoexponentially; thus, they were fitted to a 1-compartment model. A set of representative plasma concentration-time profiles of IF isomers in male and female rats is shown in Figure 3. Relevant pharmacokinetic parameters of (R)- and (S)-IF were computed and are summarized in (Table 1). As shown, in the male rats (R)-IF exhibited a shorter half-life of 34.2 minutes than (S)-IF, which gave a half-life of 41.8 minutes (P < .01). AUC and total body clearance of (R)-enantiomer were

significantly larger than those of its antipode. Nonrenal clearance of (R)-IF was higher than that of (S)-IF. However, there was no statistical difference in volumes of distribution and renal clearance between these 2 enantiomers.
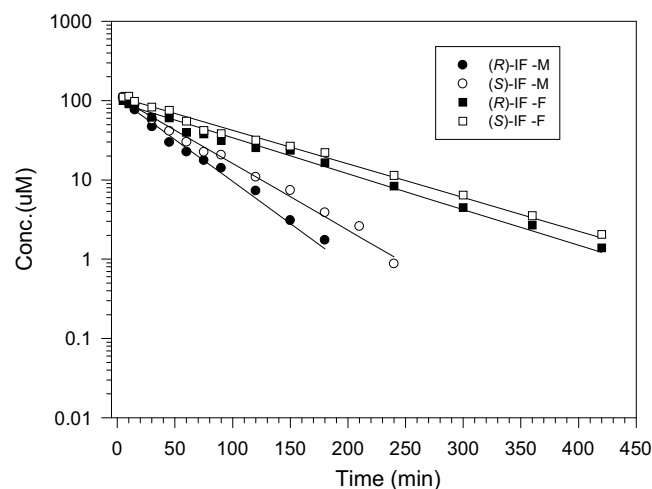

Figure 3. Representative concentration-time profiles of IF enantiomers after intravenous administration of IF pseudoracemates into male (M) and female (F) rats at a total dose of 40 mg/kg.

Table 1. Relevant Pharmacokinetic Parameters of (R)-IF and (S)-IF After Intravenous Administration of IF Pseudoracemate Into Male and Female Rats at a Total Dose of 40 mg/kg*

Pharmacokinetic Parameters		Male (n = 6)	R/S[a]	Female (n = 5)	R/S	pValue[b]
C_0 (µM)	R	99.6 ± 38.0	0.959	95.6 ± 31.6	0.946	>.5
	S	103 ± 39		103 ± 38		>.5
λ^c (min⁻¹)	R	0.0207 ± 0.0031^d	1.23	0.0114 ± 0.0019^d	1.21	<.01
	S	0.0168 ± 0.0023		0.00951 ± 0.00176		<.01
$t_{1/2\lambda}$ (min)	R	34.3 ± 5.2^d	0.820	62.1 ± 9.5^d	0.833	<.01
	S	41.8 ± 6.0		75.1 ± 14.6		<.01
MRT (min)	R	49.4 ± 7.4	0.820	89.6 ± 13.7^d	0.834	<.01
	S	60.3 ± 8.5		108 ± 21		<.01
AUC (min x µM)	R	4853 ± 1942^d	0.784	8433 ± 2480^d	0.782	<.01
	S	6259 ± 2756		10703 ± 2707		<.01
CL_T (mL/min/kg)	R	17.9 ± 6.8^d	1.29	9.66 ± 2.55^d	1.29	<.01
	S	14.0 ± 5.3		7.46 ± 1.64		<.01
$V_{d,ss}$ [e] (L/kg)	R	0.888 ± 0.385	1.05	0.863 ± 0.243	1.07	>.5
	S	0.838 ± 0.326		0.817 ± 0.254		>.5
U (% dose in 36-hour urine)	R	13.1 ± 3.7	0.934	23.9 ± 5.1^d	0.839	<.01
	S	14.2 ± 4.3		28.4 ± 4.7		<.01

*MRT indicates mean residence time; AUC, area under the curve; CL_T, total drug clearance; $V_{d,ss}$, [author to supply].
[a]R and S indicate (R)- and (S)-IF enantiomers, respectively. [b]P values represent the significance levels for the differences in the parameters between male and female groups.
[c]Terminal decay rate constant.
[d]Significantly different from the values of (S)-isomer value (comparison of vertical rows) at P < .01.
[e]Volume of distribution at steady state

In the female rat, (R)-IF exhibited a shorter half-life, higher total body clearance, and higher nonrenal clearance than (S)-IF. The renal clearance and volume of distribution were found to be the same for both enantiomers. However, the half-lives of (R)- and (S)-IF in female rats were 62.1 and 75.1 minutes, respectively, significantly longer than those in male rats. Additionally, the nonrenal clearance in the female rat was 2.1-fold smaller than that in the male rat, indicating a lower metabolic capacity in the female rat. Thus, there is a significant gender difference in metabolism of IF. It is interesting to note that the initial concentrations (C_o) and volumes of distribution were the same for 2 enantiomers in male and female rats, which strongly suggested that the difference in stereoselective pharmacokinetics of IF and its gender difference were mainly caused by the difference in IF metabolism.

Metabolite Kinetics of IF Enantiomers in Male and Female Rats

A set of representative plasma concentration-time profiles of HOIF in male and female rats is shown in Figure 4. As shown, plasma HOIF levels generated from (R)- and (S)-IF for both sexes peaked early and declined essentially in parallel to those of their respective parent drugs, indicating a formation-limited process (30). The relevant pharmacokinetic parameters of HOIF in male and female rats are shown in (Table 2). AUC, peak concentration (C_{max}), and urinary excretion of HOIF (U) showed strong enantioselectivity for the R-isomer with R/S ratios of 1.7, 1.8, and 1.9 in male rats and 1.7, 2.1, and 1.8 in female rats, respectively. Thus, these data indicated a strong substrate enantioselectivity in favor of (R)-IF for the 4-hydroxylation pathway in the rat. In addition, the generated HOIF from (R)- and (S)-IF exhibited longer half-lives and slightly lower AUCs in female rats than in male rats, consistent with the slower elimination of IF enantiomers in the female rats.

A set of representative plasma concentration-time profiles of the metabolite, N2D, in male and female rats is shown in Figure 4. As shown, after reaching the peak values, levels of N2D isomers declined monoexponentially, with half-lives significantly

longer than those of the parent drugs in both male and female rats. The relevant pharmacokinetic parameters of N2D in male and female rats are shown in (Table 2). N2D generation showed a strong enantioselectivity for the S-isomer with statistically different AUC, C_{max}, AUC_m/AUC_p, and urinary excretion. Their respective R/S ratios were 0.29, 0.38, 0.37, and 0.33 in male rats, and 0.53, 0.58, 0.67, and 0.41 in female rats, indicating a strong preference in N^2-dechloroethylation of IF for the S-configuration. Significantly longer half-lives for both (R)-N2D and (S)-N2D were found in female rats compared with male rats.
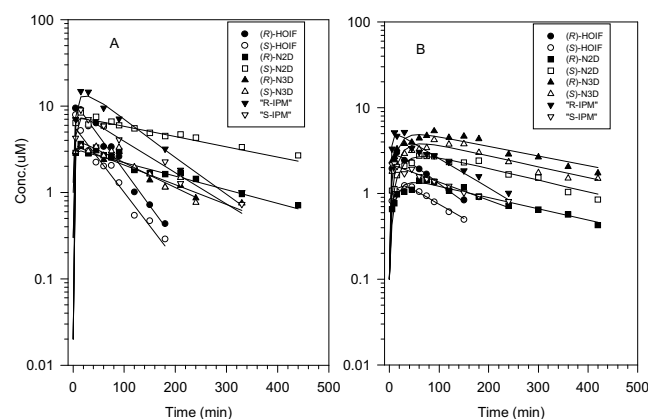

Figure 4. Representative concentration-time profiles of IF metabolites after intravenous administration of IF pseudoracemate into male (A) and female (B) rates at a total does of 40 mg/kg.

A set of representative plasma concentration-time profiles of the metabolite N3D in female and male rats is shown in Figure 4. The relevant pharmacokinetic parameters of N3D in male and female rats are shown in (Table 2). As shown, following a brief rise, N3D plasma levels declined monoexponentially, also with longer half-lives than the parent drugs. An enantioselective generation in favor of the (R)-N3D exists, giving R/S ratios of 1.4, 1.3, and 1.4 in the male rat, and 2.5, 2.0, and 2.1 in the female rat for AUC, C_{max}, and urinary excretion, respectively. Because (R)-N3D is generated from (S)-IF, the formation of N3D also shows S-enantioselectivity, similar to that of N2D in the rat. It should be noted that the notation of (R)-N3D was a result of the Cahn-Ingold-Prelog nomenclature; its spatial relationship was identical to that of (S)-N2D

and to that of the precursor (S)-IF. Like N2D, the half-life of N3D in female rats was longer than that in male rats. Based on the AUC and C_{max}, N2D levels were higher than those of N3D in male rats, whereas N3D concentrations were found to be higher than those of N2D in female rats.

Although IPM is an achiral molecule, its stereochemical origin can be tracked via deuterium labels. Thus, the "R" and "S" designations of IPM refer to those of the parent enantiomers. A set of representative plasma concentration-time profiles of IPM in male and female rats is shown in Figure 4. The relevant pharmacokinetic parameters of IPM in

the rat are shown in (Table 2). As shown, similar to HOIF, IPM formation exhibited highly significant enantioselectivity in both male and female animals for (R)-isomer with "R/S" ratios of 1.5, 1.6, and 1.5 in male rats, and 1.7, 1.9, and 1.6 in female rats for AUC, C_{max}, and urinary excretion, respectively. Because IPM is known to generate from nonenzymatic degradation of HOIF, the formation of which is highly enantioselective, the enantioselectivity of IPM generation may be derived from that of HOIF. The gender difference in IPM elimination kinetics was also observed, and the half-life of IPM in female rats was longer than that in male rats.

Table 2. Relevant Pharmacokinetic Parameters of IF Metabolites in Male and Female Rats Given IF Pseudoracemate at 40 mg/kg Total Dose*

Parameters[a]		HOIF Male (N = 6)	HOIF Female (N = 5)	N2D Male (N = 6)	N2D Female (N = 5)	N3D[b] Male (N = 6)	N3D[b] Female (N = 5)	IPM[c] Male (N = 6)v	IPM[c] Female (N = 5)
λ^d (x 10^{-3} min^{-1})	R[b]	18.1 ± 3.1	11.4 ± 2.1[e]	4.30 ± 0.67[e]	2.14 ± 1.16	3.79 ± 1.04	2.29 ± 1.31	**9.58 ± 2.48**	**7.64 ± 0.95**
	S	16.6 ± 1.4	9.50 ± 2.36[e]	3.39 ± 0.78[e]	1.97 ± 0.95	4.42 ± 1.28	2.63 ± 1.08	9.34 ± 2.87	6.33 ± 2.12
	R/S	1.09	1.22	1.29	1.06	0.864	0.857	1.04	1.28
$t_{1/2\lambda}$ (min)	R	39.2 ± 5.9	62.1 ± 10.5[e]	164 ± 24[e]	423 ± 244	196 ± 58	388 ± 199	75.7 ± 15.5	91.8 ± 10.6
	S	41.9 ± 3.6	76.4 ± 17.5[e]	211 ± 42[e]	433 ± 222	167 ± 44	313 ± 155	78.3 ± 16.1	118 ± 31
	R/S	0.935	0.825	0.791	0.953	1.18	1.25	0.974	0.83
AUC (μM* min)	R	**376 ± 116[e]**	**277 ± 65[e]**	**799 ± 207[e]**	**716 ± 358[e]**	1380 ± 519	2995 ± 893[e]	**1304 ± 288[e]**	**1485 ± 673[e]**
	S	**221 ± 60[e]**	**170 ± 54[e]**	**2794 ± 607[e]**	**1436 ± 694[e]**	**966 ± 192**	**1300 ± 519[e]**	**876 ± 177[e]**	**863 ± 321[e]**
	R/S	1.70	1.69	0.287	0.530	1.41	2.50	1.50	1.68
%AUC_m/AUC_p	R	8.77 ± 3.97[e]	3.34 ± 0.42[e]	17.4 ± 4.2[e]	9.87 ± 6.91[e]	**24.7 ± 13.4**	**29.8 ± 13.8[e]**	31.8 ± 16.4[e]	19.8 ± 12.8[e]
	S	4.02 ± 1.68[e]	1.59 ± 0.33[e]	48.5 ± 14.3[e]	14.6 ± 9.18[e]	**22.2 ± 8.6**	**16.7 ± 6.89[e]**	16.5 ± 8.3[e]	8.77 ± 4.69[e]
	R/S	2.18	2.15	0.365	0.674	1.09	1.98	1.92	2.16
C_{max} (μM)	R	5.90 ± 1.96[e]	2.73 ± 0.67[e]	2.98 ± 0.71[e]	1.11 ± 0.28[e]	4.82 ± 1.85	**5.46 ± 2.84[e]**	**11.6 ± 2.17[e]**	**8.31 ± 3.49[e]**
	S	3.38 ± 1.13[e]	1.49 ± 0.71e	7.75 ± 1.76[e]	2.11 ± 0.66[e]	3.74 ± 0.84	**2.81 ± 1.20[e]**	7.35 ± 1.67[e]	4.52 ± 2.12[e]
	R/S	1.77	2.05	0.384	0.576	1.27	2.01	1.59	1.90
U (% dose in 36-hour urine)	R	**2.12 ± 0.77[e]**	**2.88 ± 1.05[e]**	4.48 ± 0.65[e]	1.61 ± 0.42[e]	5.76 ± 1.68	11.1 ± 5.74	**29.4 ± 5.8[e]**	**35.4 ± 6.52[e]**
	S	**1.15 ± 0.36[e]**	**1.66 ± 0.64[e]**	14.0 ± 2.8[e]	4.35 ± 1.59[e]	**4.10 ± 1.06**	**5.20 ± 2.08**	**19.1 ± 2.6[e]**	**22.5 ± 4.97[e]**
	R/S	1.85	1.75	0.328	0.414	1.42	2.10	1.53	1.59

*HOIF indicates 4-hydroxyifosfamide; N2D, N^2-dechloroethylifosfamide; N3D, N^3-dechloroethylifosfamide; IPM, isophosphoramide mustard.

[a]Differences in all parameters of (R)- and (S)-metabolites between male and female rats (comparison of horizontal columns) statistically significant at P = .01, except for those indicated in bold letters.

[b](R)- and (S)-N3D are generated from (S)- and (R)-IF, respectively.

[c](R)- and (S)-IPM refer to non-chiral IPM generated from (R)- and (S)-IF, respectively.

[d]Terminal decay rate constants.

[e]Significant difference between (R)- and (S)-isomers (comparison of vertical rows) at P < .01.

In Vitro Metabolism of IF in Rat Liver Microsomes

The formation of all metabolites by microsomes showed linearity up to 2 mg microsomal protein/mL and up to 30 minutes of incubation time in male rats. (Table 3)summarizes the K_m and V_{max} values for the 3 primary metabolic pathways of each enantiomer in male and female rat liver microsomes. There were no significant differences in K_m values for individual enantiomers for each metabolic pathway, except for N^3-dechloroethlation of IF. For the latter, K_m was smaller for (R)-IF than for (S)-IF in the female rat, and the sequence of the magnitude was reversed in the male rat (P = .05). In both male and female rats,

the mean V_{max}/K_m (intrinsic clearance) values for 4-hydroxylation of (R)-IF were greater than the corresponding values of (S)-IF. In contrast, V_{max}/K_m values for N^2-dechloroethylation pathways for (R)-IF were nearly equal to those for the (S)-isomer. For N3D formation, the intrinsic clearance values were greater in the male rat than in the female rat. The overall intrinsic clearance of IF in male rat microsomes (sum of the intrinsic clearance values for (R)-IF and (S)-IF for all 3 metabolic pathways) of 2.9 was nearly 2-fold higher than that in female rat microsomes (1.6), consistent with the in vivo observation.

Table 3. Mean Michaelis-Menten Parameters for the Metabolism of IF Enantiomers in Liver Microsomes From Three Male and Three Female Rats

Gender		HOIF			N2D			N3D		
		V_{max} nmol/min/mg	K_m mmol	V_{max}/K_m[a] μL/min/mg	V_{max} nmol/min/mg	K_m mmol	V_{max}/K_m[a] μL/min/mg	V_{max} nmol/min/mg	K_m mmol	V_{max}/K_m[a] μL/min/mg
Female	R	1.41	2.02	**0.702**	0.402	3.15	**0.135**	0.770	3.39	**0.226**
	S	± 0.28	± 0.53	± 0.044	± 0.137	± 1.68	± 0.028	± 0.178	± 0.65	± 0.009
		0.706	2.55	**0.284**	0.392	2.67	**0.159**	0.514	5.11	**0.107**
		± 0.158	± 0.94	± 0.042	± 0.142	± 1.56	± 0.039	± 0.051	± 1.94	± 0.031
Male	R	2.13	2.22	**0.965**	0.524	1.88	**0.296**	2.46	6.10	**0.433**
	S	± 0.27	± 0.34	± 0.067	± 0.074	± 0.58	± 0.093	± 0.62	± 2.13	± 0.114
		1.37	2.56	**0.545**	0.604	1.87	**0.331**	0.732	2.59	**0.293**
		± 0.31	± 0.72	± 0.041	± 0.094	± 0.32	± 0.092	± 0.187	± 0.58	± 0.106

[a]*Differences in all intrinsic clearances for three primary metabolic pathways of IF between male and female rats statistically different at P = .05. See Table 2 legend for explanation of abbreviations.*

DISCUSSION

Stereoselective pharmacokinetics of IF have been found in both humans (7-11,16-17,19) and rats (15,21). It was reported that (S)-IF exhibited a shorter half-life and higher nonrenal clearance than (R)-isomer in the human (16,17). However, the volume of distribution and renal clearance between IF enantiomers in patients were not statistically different (16). In the rat, (R)-IF was eliminated faster than its antipode, and both enantiomers exhibited the same volume distribution and renal clearance (21,30). The reason for the observed stereoselective disposition of IF was not reported. Additionally, previous studies reported enantioselectivity of IF for N-dechloroethylation only, and preference for S

configuration in both humans and rats was noted (8,9,15-19). Enantioselectivity for hydroxylation was not reported. The use of pseudoracemate and GC/MS techniques enabled investigation of stereoselectivity of N^2- and N^3-dechloroethylation and 4-hydroxylation pathways of IF (21,31). The present study demonstrated that (S)-IF produced a longer plasma half-life and a higher AUC than (R)-IF. Additionally, plasma metabolic profiles were quantitatively different between the enantiomers.

Following intravenous bolus administration of a chiral drug, the manifested stereoselective pharmacokinetics could derive from stereoselective distribution, metabolism, or excretion (12-14,27). Robinson and Mehvar found that verapamil and

norverapamil were enantioselectively distributed into human and rat erythrocytes, and the stereoselective plasma protein binding was the major factor for the selective distribution (27). For IF, both enantiomers exhibited the same protein binding and erythrocyte uptake. Together with the evidence of nearly identical volume of distribution between 2 enantiomers in both male and female rats, we ruled out the possibility of enantioselective distribution of IF in the rat. Additionally, the enantiomers showed the same renal clearances in both male and female rats, indicating the absence of enantioselective excretion of IF in rats. Consequently, the observed enantioselective pharmacokinetics of IF appeared to be mainly attributed to the enantioselective metabolism. In fact, quantitation of generated metabolites revealed that 2 isomers preferentially underwent different metabolic pathways. (R)-IF was biotransformed in favor of 4-hydroxylation pathway, while (S)-isomer was preferentially metabolized through N^2- and N^3-dechloroethylation in both male and female rats. The enantioselective drug metabolism was further confirmed by the in vitro metabolic study, which showed that total intrinsic clearance (V_{max}/K_m) of (R)-IF was higher than that of (S)-IF in male or female rat liver microsomes.

A gender difference in IF pharmacokinetics was also observed in this study. Lower nonrenal clearances and longer half-lives for both IF enantiomers were found in female rats, and all of the major metabolites exhibited longer half-lives in female rats than in male rats. Because the same volumes of distribution and renal clearances were found in both male and female rats, the differential metabolism may be the only reason for the observed gender difference. In fact, a higher metabolic activity toward IF was observed in male rat liver microsomes than in female rat microsomes, consistent with the in vivo result. It has been reported that CYP2C6 and CYP2C11 are 2 isozymes responsible for IF metabolism in the rat (23,32). CYP2C6 primarily exists in the female rat liver. Both CYP2C6 and CYP2C11 are found in male rat liver, and CYP2C11 is a more active isozyme toward IF metabolism than CYP2C6 (24). Therefore, this difference may account for the gender difference of IF metabolism in the rat. Taken collectively, these findings demonstrated that stereoselective metabolism was the only causative factor to the observed gender and stereoselective difference in pharmacokinetics of IF in the rat.

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